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14. ABSTRACT
miRNAs have recently been identified as epigenetic elements that have important roles in development, differentiation, apoptosis and oncogenesis. Altered expression of several miRNAs have been reported in human breast cancers and may be useful in predicting patient prognosis. The functional roles of miRNAs in tumor development and progression have not been well evaluated. The purpose of this study is to use multiple genetically engineered mouse models of mammary cancer as a filter to identify miRNAs whose expression may be evolutionarily conserved in breast cancer. Such species of miRNA that are identified through a cross-species comparison are likely to be functionally important. This study has determined the miRNA expression in multiple mouse models of mammary cancer that are based upon different initiating oncogenic events. Four general patterns of miRNA expression have been identified among the models by hierarchical clustering analyses. A distinct miRNA expression pattern has been identified for MMTV-her2/neu tumors, another for MMTV-myc tumors, whereas p53-/- tumors cluster separately from C3(1)/Tag and MMTV-PyMT tumors. Current analyses are underway to correlate changes in miRNA expression with array CGH and gene expression data from the same tumors and compare these findings to miRNA alterations in human cancer. Functional analyses are also being performed.

15. SUBJECT TERMS
breast cancer; animal models; genomics; cross-species analysis; miRNA

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Introduction

MicroRNAs (miRNAs) are recently discovered but minimally explored non-protein-coding double-stranded RNA molecules that regulate gene expression involved in the control of development, proliferation, differentiation, apoptosis, stress response and cancer (2). Recent studies of miRNAs in numerous malignancies have demonstrated that they are frequently located at genomic regions involved in cancers, play a critical role in post-transcriptional regulation of oncogenes and tumor suppressor genes (4), and are associated with poor prognosis in leukemia, colorectal neoplasia, lung cancer, and breast cancer (1-3, 5). The functional roles of miRNAs in breast cancer etiology and tumor progression are not understood.

Relevant mouse models of human breast cancer have been successfully used as a filter to help identify evolutionarily conserved gene expression patterns in breast cancer (5). It is quite likely that genes and genetic networks that are found to be operative in both the mouse and human cancers, are critical to the development and maintenance of the tumor phenotype. Use of mouse models in conjunction with human tumor data provide a means to overcome inherent limitations of expression variance in the human population due to great genetic diversity in the population. This approach can leads to the identification of high value, functionally conserved genes. The identification of such genes also leads to the identification of candidate genes for targeted therapies.

We have demonstrated the relevance of several existing mouse mammary cancer models that can be directly clustered with three sub-types of human breast cancer using the Sorlie classification gene signatures (Green, unpublished data, and 7). This proposal uses this same principle of integrating mouse model data to identify the evolutionarily conserved, functionally most important miRNAs related to human breast cancer. By integrating studies of relevant mouse models with human breast cancer, this approach should lead to the identification of the likely miRNA candidates that are functionally important for breast cancer development and that may serve as new targets for breast cancer detection, diagnosis and therapy. Functional validation of the candidate miRNAs can be subsequently performed in in vitro and in vivo assays.

Body

Hypothesis/Rationale/Purpose.

An integrated analysis of miRNA expression in GEM mammary cancer models with that of human breast cancer will provide significant insights into the roles of miRNAs in oncogenesis. A cross-comparison of human and mouse miRNA data will identify evolutionarily conserved candidate oncogenic miRNAs that likely are functionally highly significant for breast cancer pathogenesis. These candidates can be functionally tested for their role in tumorigenesis, may serve as potential biomarkers and may identify new targets/pathways for therapies.

In order to more fully compare genomic data between mouse and human, we have additionally initiated array studies to assay a larger number of genes (than present in our older datasets) using an Affymetrix platform and array CGH to identify alterations in copy number changes in chromosomal DNA using an Agilent platform.

Experimental Approaches.

Collection of mammary tumors from mouse models.

Multiple tumor samples were collected from several animal models including C3(1)/Tag, MMTV-her2/neu, MMTV-myc, MMTV-PyMT; $p53^{-/-}$ ER+ transplant model, $p53^{-/-}$ ER- transplant model, and BRCA1 ; $p53^{+/-}$ model. MMTV-ras tumors were not initially available and animals have been bred to generate these tumors for inclusion into the study.

Isolation of miRNA, mRNA and DNA from the tumor samples.

Total RNA was isolated from the tumor samples using a method that did not exclude small, miRNA species. This allowed for the use of the total RNA for both miRNA and expression profile arrays. DNA was also extracted from the same tumor samples for array CGH analyses. RNA and DNA were quality controlled using an Agilent Lab-on-a-Chip Bioanalyzer. Samples with poor quality were excluded from analyses and have been or will be replaced to fully populate the datasets.

miRNA array analyses

Total RNA from tumor samples were labeled with cy-3. Ambion mouse reference RNA was used as the standard reference RNA since it is produced in a manner that captures the miRNA species and was labeled with cy-5. Initially, we evaluated several miRNA platforms to determine which platform would produce the best results. We found that the Ambion miRNA platform was not as sensitive as other platforms and therefore decided to conduct all analyses using an Agilent-produced array that was designed by the Frederick miRNA array core. This platform was designed as a 2-channel platform that gives highly reproducible results with good sensitivity and specificity. Results of miRNA analyses are provided in Figure 1 and Figure 2.

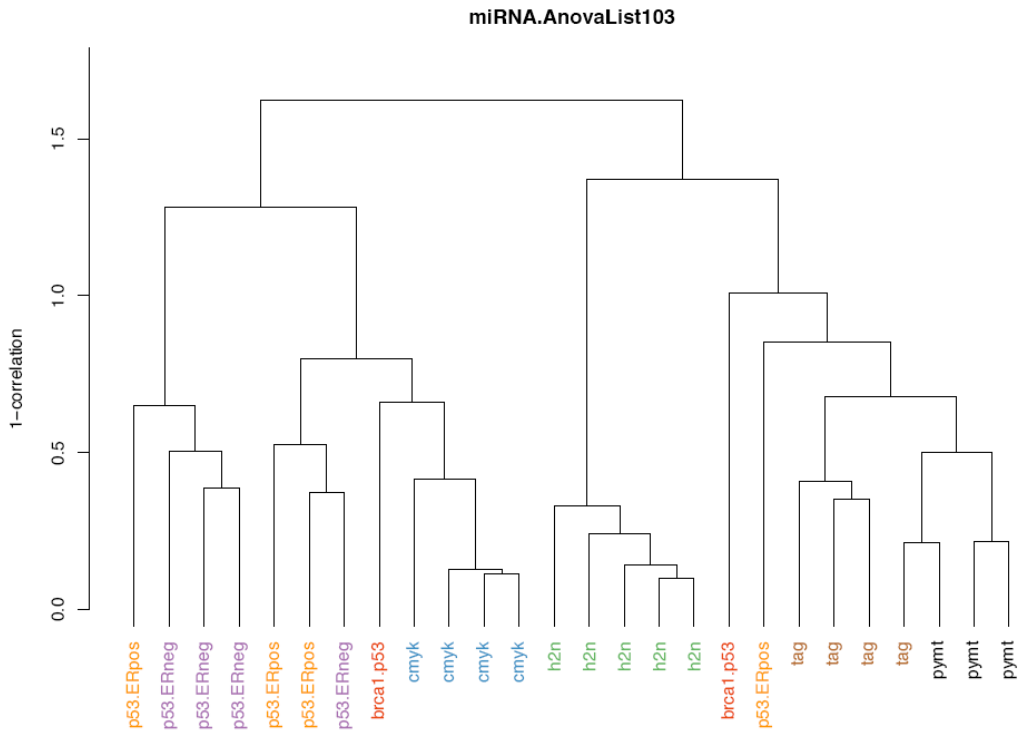

Figure 1.
Differentially expressed genes were identified with one-way ANOVA (univariate F-tests) across all models (7-level factor). The genes differentially expressed between the models were found with the alpha level cutoff at 0.01. Hierarchical clustering: the 103 genes were median centered and the 1 minus Pearson correlation distance with complete linkage were applied to generate the dendrogram.

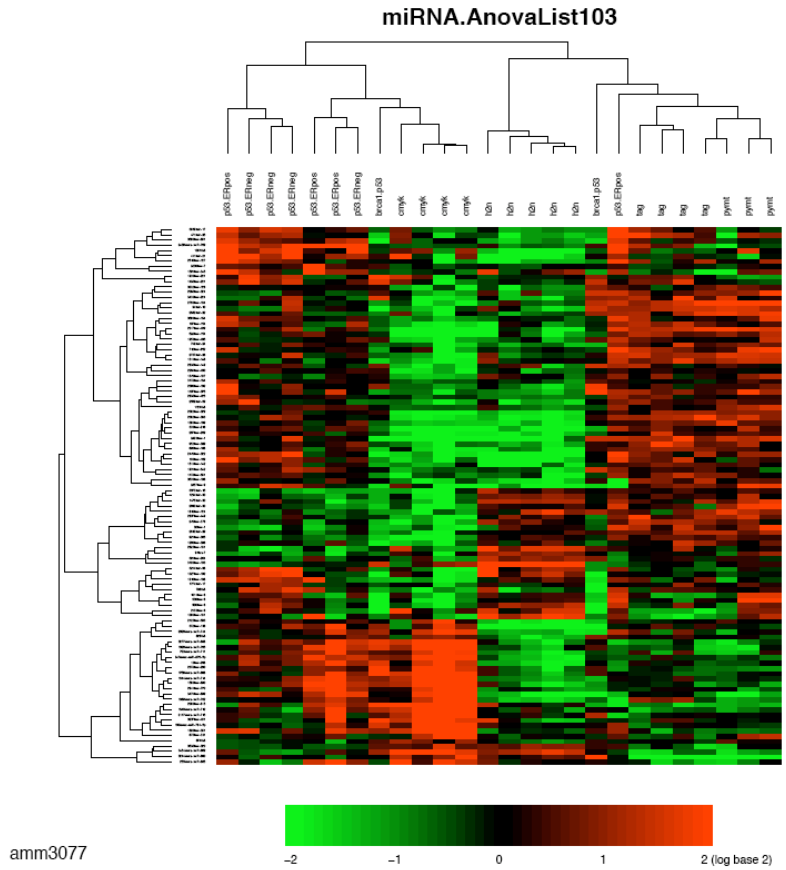

Figure 2.
Hierarchical clustering: the 103 genes as described in Figure 3 were median centered and the 1 minus Pearson correlation distance with complete linkage were applied to generate the heatmap. The clustering shows that her2 and c-myc have the most defined miRNA expression profiles (within cluster correlation around 0.6).

Expression profiling analyses

All tumor samples were analyzed using the Affymetrix U130 array which provided significantly more gene representation than data previously generated in our lab, thus providing additional data to correlate with the miRNA data. Since the targets of miRNAs are poorly understood and have generally only been identified by computational methods, but not validated biologically, this additional level of information will be valuable in selecting miRNA targets for further validation. Data from these studies indicate that the tumor models cluster into separate categories as expected (Figures 3, 4 and 5). The clusters based upon expression can be compared with the clusters identified by miRNA array analyses.

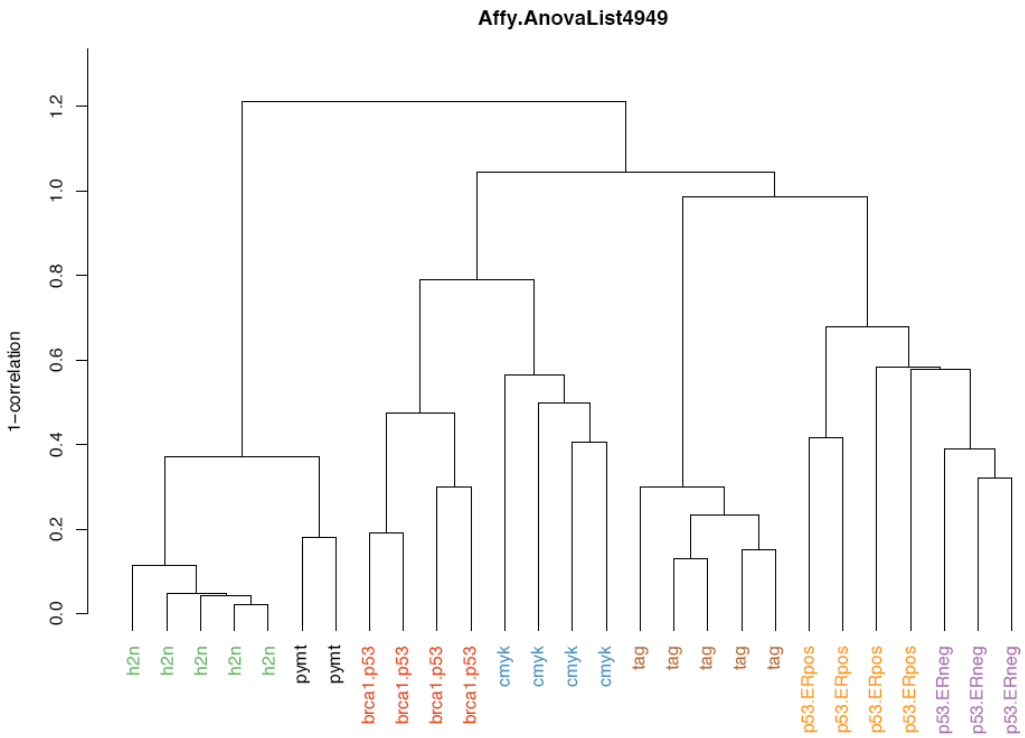

Figure 3.

Differentially expressed genes were identified with one-way ANOVA (univariate F-tests) across all models (7-level factor). The genes differentially expressed between the models were found with the alpha level cutoff at 0.001. Hierarchical clustering: in each case the genes were median centered and the 1 minus Pearson correlation distance with average linkage were applied to generate the dendrograms. Gene selection: 4949 probes to generate sample dendrogram.

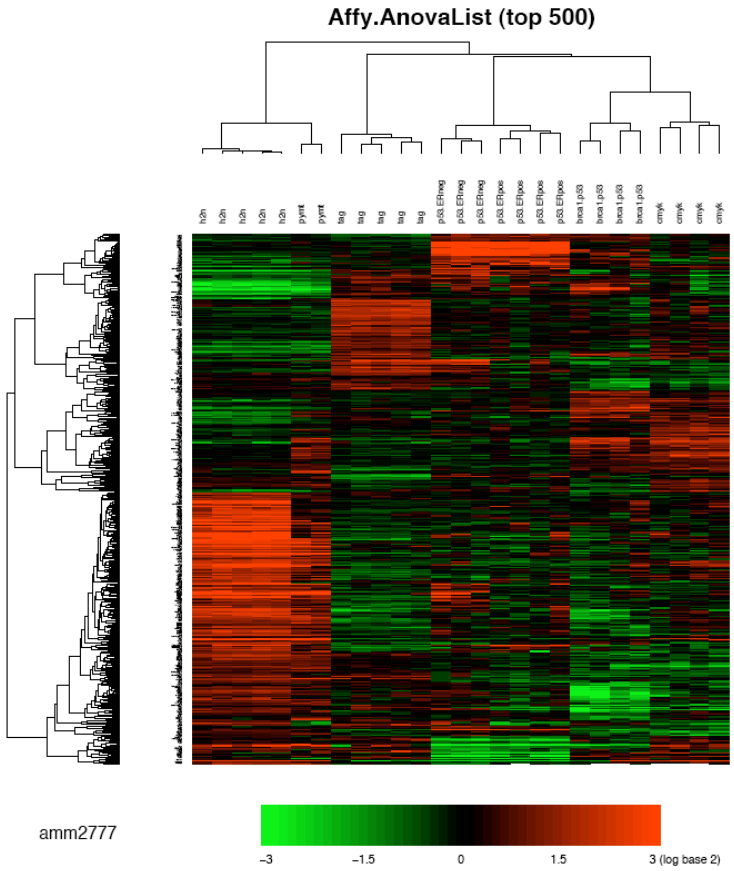

Figure 4.
500 top genes (p value rank) to generate two-way clustering heatmap. The hierarchical clustering analysis shows clear model-specific signatures, which are robustly preserved across the gene selections described above.

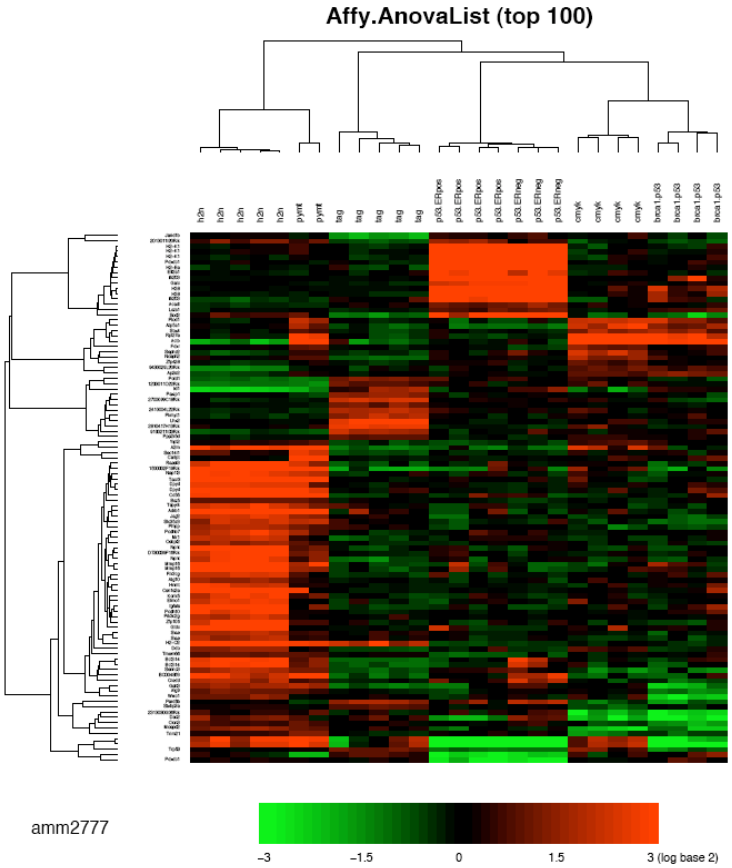

Figure 5.
500 top genes (p value rank) to generate two-way clustering heatmap.

Array CGH analyses

DNA from all tumor samples has been used to perform 2-channel array CGH studies using the high density Agilent mouse CGH array. It is clear that alterations in genome copy number vary between the models and this may be related to changes in miRNA expression and gene expression. See appendix for representative information.

Functional analyses and bioinformatics analyses

The complexity of genomic alterations in the models studied have led to significant informatics challenges that we are currently addressing. No tools are readily available that allow us to cross-compare expression profiles with miRNA profiles and array CGH data together. We are working on methods to improve these types of analyses. This has delayed our completion of identifying all of the miRNA candidates we will functionally test for their role in the development and progression of breast cancer. This work remains ongoing and a high priority.

Key Research Accomplishments:

1) collection of tumors from multiple mouse models of human breast cancer initiated by different oncogenic events;
2) determination of miRNA expression from these models and cross-comparison of expression between the models;
3) determination of gene expression profiles from the same tumors and cross-comparison of the expression profiles between the models;
4) determination of genome-wide copy number alterations using array CGH and cross-comparisons between the models;

Reportable Outcomes:

1) miRNA expression patterns are quite distinct between different models dependent upon the initiating oncogenic event;
2) MMTV-her2 and MMTV-myc models appear to have the least number of alterations in miRNA expression patterns;
3) The alterations in miRNA expression in the MMTV-her2 and MMTV-myc models are generally distinct from those observed in the C3(1)/Tag, MMTV-PyMT, p53-/- and BRCA1-/-;p53+/- models;
4) Specific miRNAs may tend to cluster together suggesting that there may be patterns of miRNA changes that need to be explored in a systems approach;
5) Specific alterations in oncogene expression or loss of specific suppressor gene function may determine the expression pattern of miRNAs.

Conclusions:

The results from this study provide a robust dataset of genomic information at three levels - miRNA, mRNA, and genome copy number - from mouse models of mammary cancer for comparison with that of human breast cancer. Analyses of the data indicate that miRNA patterns are quite distinct between the models of mammary cancer dependent upon the initiating oncogenic event. This may in part be due to the cancer precursor cell that is being targeted by the manner in which the genetic alteration has been engineered in the mouse germline, or it may be due in part to the particular function of the oncogene or suppressor gene used to generate the tumors. It is possible that part of the difference in miRNA expression between the models is therefore determined by the lineage through which the tumor arises. For instance, MMTV-her2/neu and MMTV-myc may arise through the targeting of tumorigenesis to precursor cells destined to become luminal type cells, whereas models generated through the disruption of p53 and/or BRCA1 function may target a more primitive precursor cell. Differences in cell lineage could also account for changes in the mRNA expression pattern.

Ongoing efforts are focused on further identifying the major similarities in miRNA expression observed in the mouse mammary tumors with that reported for human breast cancer. miRNAs meeting this criteria will be further studied in appropriate in vitro and in vivo analyses.

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Genome Biol. 2007 May 10;8(5):R76

Appendices

	chr 1		chr 2		chr 3		chr 4		chr 5	
Sample	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)
c-myc Tu 4 An:7										
c-myc Tu 043508					3.55-86.05, 101.35-159.8					
c-myc Tu 04004021					whole chr			42 - 96.1		
c-myc Tu 04004022					whole chr					
c-myc Tu 04005648					3.4-82.1			whole chr		
MMTV-PyMT 317					66.293					
MMTV-PyMT 341					66.293					
MMTV-PyMT 342								119.775		
MMTV-PyMT 427					66.293			119.775		
MMTV-PyMT 435					66.293			119.775		
C3/TAg 2					45.3-45.6		56.7-81.85			
C3/TAg 4					45.3-45.6					
C3/TAg 5					45.3-45.6					
C3/TAg 7	4.7-72.85				45.3-45.6					
C3/TAg 8					45.3-45.6		56.7-81.85			
MTV-H2N Founder A				whole chr					whole chr	
MTV-H2N 1				whole chr LOH					whole chr	
MTV-H2N 53				whole chr LOH					whole chr	
MTV-H2N 61									whole chr	
MTV-H2N 64			whole chr		whole chr, gain of 1 copy				whole chr	
p53-/- 5809R_PN2(254c)					whole chr					
p53-/- 5817_PN2(254c)										
p53-/- 5851L_PN2(254c)					whole chr					
p53-/- 2979R_PN1b					whole chr					
p53-/- 1573R_PN1b	whole chr, gain of 1 copy		whole chr, gain of 1 copy		3.415-28.9, 117.7-159.8					
p53-/- 8546R_PN1b	whole chr, gain of 1 copy		whole chr, gain of 1 copy		3.415-28.9, 117.7-159.8					

chr 6		chr 7		chr 8
Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)
		81.4 - 118.45		
		54.75 - 115.8		
		50.4 - 118.45		
		139-141.05, 141.4-142.1 gain of more than 2 copies		
			18.1-45.45	
			15-50.35	
			18.4-29.8	
139.8-140.7, 141.2-142.3, 142.3-149.45 Gain of 2 to 4 copies			whole chr, LOH	
whole chr			whole chr, LOH	
			whole chr, LOH	
126-131, 132-132.5, 133.5-134.5, 135.1-137.1, 136.9-138.6, 139.5-142.75, 143.1- 149.45			28.1-145.1	
Gain of 1 to 4 copies. See PowerPoint presentation				
3.25-99.75, 139.5-141.8, 142.1-149.45 Gain of 1 to 4 copies. See PowerPoint presentation			33.85-40.8, 41.65-79.3	
whole chr				
15.3-18.75 gain of 4 copies	43.2-146.4			
8.455-12, 16.7-21.65 gain of 4 copies	21.9-146.4			
whole chr				
whole chr, gain of 1 copy, 17.4-21.3, 22.9-25.7 gain of 4 copies				
whole chr, gain of 1 copy, 17.2-17.9 gain of 4 copies				

	chr 9		chr 10		chr 11		chr 12	
Deletion (Mb)	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)
67.8 - 129.1		whole chr			103.7-121.6 gain of 1 copy			
		start of chr - 98.75	106.4-108.35, 116.3-117.75, 118.9-129.7		112.4-121.6 gain of 2 copies			
			gain of one copy in the 3 areas of amplification					
72.65 - 87.9								
72.65 - 108.8					105.7-105.8, 110.85-113.25, 114.15-117.6			
					gain of 2 copies			
							29.2-86.45	
					whole chr			
					whole chr			
							52.2-86	
72.65-129.1								
73.75-87.95								
				whole chr, LOH		whole chr		
				whole chr, LOH		whole chr		
				whole chr, LOH		whole chr		
				whole chr, LOH				
				9.505-57.7				

chr 13		chr 14		chr 15		chr 17	
Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)
		6.8-22.9, 31.75-52.7					
		whole chr					
				16.7-77.15			
				whole chr			
				whole chr			
				16 gain of 1 copy			
				16 gain of 1 copy			23-47
				16 gain of 1 copy			
				16 gain of 1 copy			23-34
				16 gain of 1 copy			
						whole chr	
	whole chr, LOH					whole chr	
	whole chr, LOH					5.25-23.4, 46.3-55.5, 56.9-83.7	
whole chr						whole chr	
	8.725-71.2					whole chr	
whole chr, LOH, 114.8-115 gain of 4 copies							
4.04-35.65, 60.75-91.7, 97.4-111.3, 114.8-115 gain of 4 copies							
whole chr, LOH							
whole chr, 114.8-115 gain of 4 copies							
whole chr, 114.8-115 gain of 4 copies							

chr 18		chr X	
Amplification (Mb)	Deletion (Mb)	Amplification (Mb)	Deletion (Mb)
	10.9 Possible homozygous deletion		
	10.9 Possible homozygous deletion		
		whole chr, gain of 2 copies	
whole chr, gain of 1 copy		gain of more than 1 copy	
	whole chr		
21.15-34.6			
whole chr, gain of 1 copy		more than 2 copies in some areas	
whole chr		more than 2 copies in some areas	